Stereospecificity in the enzymatic hydrolysis of cyclosarin (GF)

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Abstract

Enzymatic catalysis is one means of accelerating the rate of hydrolysis of G-type organophosphorus nerve agents. Here, the stereospecificity of the catalysis of cyclosarin (GF, O-cyclohexyl methylphosphonofluoridate) hydrolysis by several enzymes was investigated. Stereospecificity was not evident at 3 mM GF but was evident at 0.5 mM GF. The differential effect was apparently due to fluoride-catalyzed racemization of the substrate. Alteromonas sp. JD6.5 organophosphorus acid anhydrolase (OPAA), Alteromonas haloplankis OPAA and the wild-type phosphotriesterase (PTE) enzymes were all found to catalyze preferentially the hydrolysis of the (+)GF isomer, as determined by GC analysis of the remaining unreacted (−)GF isomer. Acetylcholinesterase inhibition experiments showed the purified (−)GF isomer to be approximately twice as toxic as the racemic mixture. One PTE mutant, H254G/H259W/L303T, was found to reverse the native PTE stereospecificity and preferentially catalyze the hydrolysis of the (−)GF isomer, as shown by its complementation of Alteromonas sp. JD6.5 OPAA and by GC analysis of the remaining (+)GF isomer. This procedure also permitted the individual preparation of either of the two GF isomers by enzymatic degradation followed by extraction of the remaining isomer.

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1. Introduction

The organophosphorus acid anhydrolase (OPAA), from Alteromonas sp. JD6.5 has been shown to catalyze the hydrolysis of a number of toxic organophosphorus compounds including several G-type chemical nerve agents, the generalized structure of which is shown in Fig. 1 [1,2]. Its gene has been cloned into Escherichia coli and the enzyme can be produced at concentrations up to 300 mg per liter of culture, corresponding to approximately 50% of the total cellular protein [3]. The gene encoding a similar OPAA enzyme from Alteromonas haloplankis has also been cloned and expressed in E. coli [4].

The phosphotriesterase enzyme (PTE) has some catalytic properties similar to OPAA. In addition, PTE possesses unique catalytic activity against the nerve agent VX (O-ethyl-S-(2-diisopropylaminocetyl) methylphosphonothioate. In nature, the PTE gene was found on dissimilar-sized plasmids in two soil bacteria, Pseudomonas diminuta MG and Flavobacterium sp. ATCC 27551 [5–7]. The gene has been cloned and sequenced [6–8] and the enzyme has been overexpressed in several systems [9–11]. PTE catalyzes the hydrolysis of a broad spectrum of organophosphorus compounds including those with P=O, P=S, P=CN and P=N bonds to their leaving groups [9,12–15]. Recently, a number of site-directed mutants derived from PTE have also been characterized with respect to their activity against various organophosphorus compounds [16–18].

While several reports have described the initial rate kinetics of these enzymes on various chemical nerve agent substrates, relatively little attention has been paid to the stereospecificity of those reactions [3,12,13,15–18]. This issue is important for two reasons: First, it is possible that the differential activity on respective stereoisomers could affect the overall enzymatic detoxification rate (for this reason, it needs to be determined whether all, or at least the most toxic stereoisomers of a particular chemical agent are effective
substrates for these enzymes, and under what conditions), second, it is possible that enzymatic stereospecificity could be exploited to produce higher value products through synthetic routes. Single enantiomer compounds play a critical role as biologically active compounds and hence the stereospecific and substrate-specific nature of enzymes makes them a good choice as catalysts in the synthesis of pharmaceuticals and other fine chemicals.

Chemical nerve agents and enzymes both exert their effects in a biological, chiral environment, so it might be expected that the enzymatic effects on the agents would be stereospecific. This specificity was first reported in 1955 by Michel [19], who observed a biphasic inhibition of acetylcholinesterase (AChE) with GB (O-isopropyl methylphosphonofluoridate). It is known that the chemical nerve agents GB, GD (O-pinacolyl methylphosphonofluoridate), GA (ethyl N,N-dimethylphosphoramidocyanidate) and VX all bind AChE stereospecifically, resulting in a significant difference in toxicity between their respective enantiomers (Table 1). The data from Table 1 can be briefly summarized as follows: For GD, the P(−) isomers are almost solely responsible for the compound’s toxicity. For GB, the P(−) isomer is approximately twice as toxic as racemic GB, indicating that essentially all the GB toxicity is associated with the P(−) isomer. For VX, the (−) isomer is approximately 13 times more toxic than the (+) isomer, while the GA (−) isomer is approximately seven times more toxic than the GA (+) isomer.

In the case of GF, the toxicity of the individual isomers has not to our knowledge been reported. The intravenous toxicity of racemic GF though, has been reported as 53.0 μg/kg (rats), 15.3 μg/kg (rabbits) and 9 μg/kg (goats) [25]. These findings suggest that the rat toxicity of racemic GF is comparable to the mouse toxicity of racemic GB and GD.

The objective of this work was to investigate the stereospecificity of the Alteromonas sp. JD6.5 OPAA, A. halo- planktis OPAA and wild-type PTE enzymes with GF as their substrate. We also sought to develop a chromatographic means to separate the two GF isomers for analytical purposes, to measure their relative AChE-inhibiting activity, and to specifically produce individual isomer preparations through the enzymatic degradation of the contrasting isomer. Finally, the stereospecific GF activity of a series of site-specific PTE mutants was investigated.

2. Materials and methods

2.1. Enzymatic assays

Reactions were conducted in a temperature-controlled vessel in a total volume of 2.5 ml. Buffering was provided by 50 mM bis-tris-propane at pH 7.2. MnCl₂ was added to the buffer to a final concentration of 1 mM for OPAA assays. CoCl₂ was added to a final concentration of 1 mM for PTE assays. Fluoride measurements were made with a fluoride electrode attached to a Fisher Accumet 925 meter attached to a computer.

2.2. Gas chromatography method for separation of GF isomers

A Hewlett-Packard model 6890 GC equipped with a flame photometric detector in the phosphorus mode and a 25 m × 0.25 mm i.d. × 0.12 μm Chirasil-Val-L column (Chrompack) was used to analyze the GF stereoisomers. The oven temperature was 90 °C (isothermal) and the inlet and detector temperatures both were 200 °C. The injection volume was 1.0 μl with a 100:1 split ratio. The carrier gas was helium with a 1 ml/min flow rate. Under these conditions the (−)GF and (+)GF peaks were separated by 0.15 min at a retention time of approximately 8 min.

2.3. Polarimetry

Specific rotation of the single GF isomer was calculated based on measurements of the observed rotation made at 589 nm (sodium line) using a Perkin Elmer 141 Electronic Polarimeter and a sample cell with a path length of 10 cm.

2.4. AChE inhibition assays

AChE enzyme inhibition assays were conducted as described previously [26], with minor modifications. The sub-
strate 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was ob-
tained from Aldrich Chemical Co. (Milwaukee, WI). DTNB
was dissolved in 100 mM potassium phosphate buffer at
pH 7.0 to a final concentration of 0.01 M. AChE was pur-
chased from Sigma Chemical Co. (St. Louis, MO). Acetylth-
iocholine (a chromagenic AChE substrate) was purchased
from Aldrich as an iodide salt and was 98% pure.

AChE solutions were prepared at 10 ng/ml in water and
split into two 10 ml fractions. To one fraction was added 1
μl of a 10^−9 dilution of GF (either racemic or the chromato-
graphically pure single isomer) in isopropanol. The other
fraction (uninhibited) received no GF. Aliquots were removed
in triplicate from both fractions and analyzed to assess the
level of enzymatic activity on acetylthiocholine by spec-
trophotometric measurements at 405 nm.

2.5. Construction of mutant PTE strain

The site-directed mutant PTE strain was constructed using
the method of overlap extension [27] and cassette insertion
with a synthetic gene [28].

2.6. Enzyme preparation

The recombinant A. haloplanktis OPAA enzyme was pre-
pared according to the method described by Cheng et al. [30].
Briefly, the cells were grown to mid-log phase in Luria-Bertani (LB) broth and in-
duced for 6 h with IPTG. Ammonium sulfate fractionation
(40-65% saturation) yielded ∼75% pure enzyme. Further
purification through Q-Sepharose yielded an enzyme with
90–95% estimated purity.

The recombinant Alteromonas sp. JD6.5 OPAA was pre-
pared according to the method described by Cheng et al. [30].
Briefly, the E. coli host cell containing the cloned OPAA
gene was grown to late log phase in Luria-Bertani (LB) broth in a
bioreactor. Cells were harvested and resuspended in 10 mM
bis–tris–propane, pH 7.8. The enzyme was then purified by
ammonium sulfate fractionation. The 40–65% ammonium
sulfate pellet was redissolved, dialyzed and loaded onto a
10 ml Q Sepharose column. The enzyme was eluted from the
column with a linear gradient of 0.2–0.6 M NaCl. Subsequent
polyacrylamide gel electrophoresis of the pooled active pro-
tein peaks produced a single band, indicating a high degree
of purity.

Cells of the E. coli XL1 strain harboring pVSEOP7 (our
unpublished results) were used to produce the PTE en-
zyme. The pVSEOP7 plasmid contains a full-length opd
gene cloned in the expression vector, pSE420 (Invitrogen
Corp., CA, USA). The cells were grown in 1 l batches to
early log phase in LB broth in 6 l Erlenmeyer flasks contain-
ing 100 μg/ml ampicillin at 30 °C. After induction through
the addition of IPTG to 0.6 mM, cells were grown an ad-
nitional 14 h and cobalt chloride was added to 1 mM. Cells
were harvested 4 h after cobalt addition, by centrifugation,
and suspended in 10 mM bis–tris–propane, pH 7.8. Pellets
were frozen and stored at −40 °C before lysis by two passes
through a French press. The cell-free extract supernatant was
collected following centrifugation at 27,000 relative centrifu-
gal forces for 45 min.

The native PTE enzyme was purified from the super-
natant using a single strong cation exchange resin (SP-
Sepharose). The fractions containing the PTE activity were
pooled, concentrated, and dialyzed against 10 mM BTP, pH
7.8 containing 50 μM cobalt chloride. The purified enzyme
was analyzed through native acrylamide gel electrophores-
esis and estimated to be 85–90% homogeneous. The pro-
tein content was determined using Coomassie Protein Assay
Reagent (Pierce, Rockford, IL, USA) using BSA as a standard
protein.

3. Results

3.1. Assays—theory

Fluoride electrode assays offer a convenient means to
determine an enzyme’s relative activity on the different
stereoisomers of an organophosphofluoridate substrate. If de-
fluorination activity is approximately similar on all stereoiso-
mers, the shape of the plot of free (released) fluoride versus
time will approximate that obtained by base-mediated hy-
drolysis, which works equally on all isomers. If half the iso-
mers are degraded significantly more rapidly than the others,
there will be a midpoint deflection in the slope of the line
when approximately half the initial substrate concentration
has reacted.

3.2. Alteromonas sp. JD6.5 OPAA catalytic hydrolysis of
racemic GF: activity at 3 mM versus activity at 0.5 mM

Data illustrated in Fig. 2 show the results of Alteromonas
sp. JD6.5 OPAA catalysis of 3 mM GF. The monophases
curve is consistent with that of an enzyme that possesses sim-

![Fig. 2. Activity profile of the Alteromonas sp. JD6.5 OPAA enzyme with
3 mM GF. The monophase curve is consistent with equal catalysis of both
GF isomers.](image-url)
Fig. 3. Activity profile of *Alteromonas* sp. JD6.5 OPAA with 0.5 mM GF. The curve shows a distinct midpoint deflection, consistent with differential activity on the two isomers.

Fig. 3 shows the activity of *Alteromonas* sp. JD6.5 OPAA on 0.5 mM GF. At this concentration, the curve exhibits a distinct midpoint deflection with the slope of the second part of the curve approaching that of the spontaneous hydrolysis (i.e., half the substrate is hydrolyzed significantly more rapidly than the other half).

In 1965, Christen and Van den Muysenberg observed a biphasic hydrolysis of low GB concentrations in rat plasma [31]. When they did the same experiment with diisopropylfluorophosphate (a symmetrical molecule) the hydrolysis curve was monophasic. Also, when they did the experiment with higher GB concentrations the curve was monophasic. They hypothesized that free fluoride had catalyzed the racemization of the higher concentration of GB. This was supported by their observation that the addition of NaF to the plasma along with low concentrations of GB, again yielded a monophasic hydrolysis curve.

To determine if GF behaved similarly when its hydrolysis was catalyzed by *Alteromonas* sp. JD6.5 OPAA, NaF was added to a final concentration of 1 mM in the enzymatic reaction containing 0.5 mM GF. This addition of NaF caused a marked reduction in the midpoint deflection of the curve upon the addition of NaF to the reaction. The addition of NaCl to a final concentration of 1 mM in the same reaction caused no significant change in the reaction profile. These results (not shown) are consistent with fluoride-catalyzed racemization of GF.

3.3. Effects of GF concentration on stereospecificity

The effect of various higher GF concentrations up to 3 mM was examined in a series of reactions, each with 2 μg/ml PTE. A gradual attenuation of the midpoint deflection in the curve was observed, with a corresponding increase in the starting substrate concentration. Results were again consistent with fluoride-catalyzed racemization of GF.

3.4. Gas chromatographic separation of GF isomers

In order to independently analyze the two GF stereoisomers, an isothermal gas chromatographic (GC) method was developed (Section 2.2). The two GF stereoisomers were consistently separated by 0.15–0.2 min at retention times between 8 and 9 min, depending on the flow rate of the carrier gas. When racemic GF was injected, the area of the first peak was always slightly greater than half the area of the second peak (Fig. 4a).

3.5. Enzymatic preparation and polarimetry analysis of a single (−)GF isomer

A chromatographically pure (−)GF isomer was prepared by specifically degrading the isomer for which the enzyme had the greater activity. The *Alteromonas* sp. JD6.5 OPAA enzyme reaction was conducted at 15 °C and pH 7.0 to minimize spontaneous hydrolysis. At a point slightly past the midpoint deflection in the reaction profile, the solution was extracted with dichloromethane and the extract was analyzed by GC. A single GF isomer peak was observed with a retention time corresponding to the second GC peak (Fig. 4b).

The single isomer preparation was concentrated approximately 10-fold by evaporation of the solvent at room temperature. The specific rotation was measured by polarimetry at −19.3°. Therefore, the enzymatic preparation was enriched for the (−)GF isomer, indicating the *Alteromonas* sp. JD6.5 OPAA enzyme specifically degraded the (+)GF isomer.

3.6. Fluoride-catalyzed racemization versus allosteric alteration of the enzyme

Although the results described above from the addition of NaF to the enzymatic reaction are consistent with fluoride-catalyzed racemization, an alternative explanation could be that fluoride was allosterically altering the enzyme and thereby changing its stereospecific properties. In order to determine if the racemization could proceed in the absence of enzyme, a solution of this single (−)GF isomer in dichloromethane was added at 50% volume to 2 M NaF, in the absence of enzyme. Subsequent GC analysis showed two peaks, consistent with NaF-catalyzed non-enzymatic racemization of GF. Therefore, the presence of fluoride is both a necessary and sufficient condition for racemization, with or without enzyme.

3.7. Complementation test to determine stereospecificity of three enzymes

*Alteromonas* sp. JD6.5 OPAA, *A. haloplanktis* OPAA and wild-type PTE enzymes were tested individually and in combination to compare their stereospecificity on 0.5 mM GF. As shown in Fig. 5, the individual enzyme reactions all produced
Fig. 4. (a) Gas chromatogram of 90 °C isothermal separation of GF isomers. (b) GF following OPAA degradation of one isomer—only the second peak remains.

<table>
<thead>
<tr>
<th>Peak # Ret Time</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  7.845</td>
<td>37.40245</td>
</tr>
<tr>
<td>2  8.003</td>
<td>62.59755</td>
</tr>
</tbody>
</table>

Fig. 5. Biphasic fluoride release with PTE, Alteromonas sp. JD6.5, OPAA and A. haloplanktis OPAA, each alone and in combination. No complementation was evident, indicating that all three enzymes exhibited a marked preference for the same GF stereoisomer.

Similarly biphasic profiles. A complementation test was used to determine if these enzymes were all acting primarily on the same isomer or if one enzyme had primary activity on a different isomer than the other two. If any two enzymes tested in combination had significantly different stereospecificity, the shape of the curve should have tended towards monophasic. Experimentally, the profile of the three-enzyme reaction was essentially indistinguishable from the individual enzyme reaction profiles. Lacking complementary activity, it was evident that all three enzymes were primarily active on the same isomer. Since the polarimetry data established that the preference of the Alteromonas sp. JD6.5 OPAA enzyme was for the (+)GF isomer, it was therefore concluded that the PTE and A. haloplanktis OPAA enzymes also exhibit preferential activity on the (+)GF isomer.
and in the absence of GF. Fig. 6 shows the results of the AChE enzymatic preparations of chromatographically pure (+)GF isomer. The availability of racemic GF, chromatographically pure (+)GF, and from the second portion of the curve was estimated at 2.9 μmol/min/mg. Therefore, compared to the values for the native enzymes shown in Table 2, the activity on the (+)GF isomer has changed very little while the activity on the (−)GF isomer has been reduced by approximately two orders of magnitude. The estimated (+)/(−) activity ratio of the mutant enzyme is 0.16, as compared to 2.15 for the native PTE, indicating a reversal of stereospecificity as compared to wild-type. The estimated (+)/−GF isomer, a number of PTE mutants were screened. Most of these mutants showed the same preference as the wild-type enzyme or had very low levels of activity overall. The PTE mutant H254G/H259W/L303T (GWT) was of particular interest, since that mutant’s stereospecificity toward p-nitrophenyl derivatives of G-type nerve agents was known to be reversed from that of the wild-type enzyme (p-nitrophenyl derivatives have the fluoride leaving group of G-agents substituted with a p-nitrophenyl leaving group to allow for colorimetric detection of the hydrolysis product) [28]. Fig. 7 shows the GF hydrolysis profiles for the GWT mutant and Alteromonas sp. JD6.5 OPAA individually, and for the two enzymes combined. Each enzyme alone exhibits a midpoint deflection but the combination of the two enzymes clearly shows complementation (monophase catalysis). Therefore, both enzymes exhibit stereospecificity and their stereospecificity is opposite each other, meaning the GWT mutant is preferentially catalyzing hydrolysis of the (−)GF isomer. The specific activity on each isomer was estimated as in Section 3.8. The specific activity from the first portion of the curve (corresponding primarily to catalysis of the (−) isomer) was estimated at 2.9 μmol/min/mg and from the second portion of the curve was estimated at 0.5 μmol/min/mg. Therefore, compared to the values for the native enzymes shown in Table 2, the activity on the (−)GF isomer has changed very little while the activity on the (+)GF isomer has been reduced by approximately two orders of magnitude. The estimated (+)/(−) activity ratio of the mutant enzyme is 0.16, as compared to 2.15 for the native PTE, indicating a reversal of stereospecificity as compared to wild-type. 3.11. Enzymatic preparation and polarimetry analysis of a single (+)GF isomer

A chromatographically pure (+)GF isomer was prepared by specifically degrading the (−) isomer, on which the GWT enzyme had the greater activity. Slightly past the midpoint...
Fig. 7. Complementation of GF stereochemistry. PTE mutant H254G/H259W/L303T (GWT) and *Alteromonas* sp. JD6.5 OPAA. Enzyme concentration of *Alteromonas* sp. JD6.5 OPAA is five-fold lower than GWT due to its overall higher level of activity.

deflection of the reaction profile, the solution was extracted with dichloromethane and the extract was analyzed by GC. In the chromatogram, a single GF isomer peak was observed with a retention time corresponding to (+)GF. Results are shown in Fig. 8a (racemic GF control) and Fig. 8b (enzymatic preparation).

The single isomer preparation was concentrated approximately 10-fold by evaporation at room temperature and the specific rotation was measured at +5\(^\circ\). Therefore, the enzymatic preparation was enriched for the (+)GF isomer, further confirming that the GWT enzyme specifically degraded the (−)GF isomer.

4. Discussion

The data presented here demonstrate for the first time the stereospecificity of the *Alteromonas* sp. JD6.5 OPAA, *A. haloplanktis* OPAA and the PTE enzymes (including the GWT mutant) in the catalytic hydrolysis of GF. Complementation assays specifically showed the result of protein engineering of the PTE enzyme to reverse the stereospecificity and accomplish the preferential degradation of the (−)GF isomer, which was the isomer shown here to possess the greatest AChE-inhibiting activity. Given that almost all the toxicity of GA, GB, GD and VX is derived from the (−) isomers, and that the AChE-inhibition of the (−) GF isomer contributes most, and possibly almost all, of the toxicity to the racemic compound. This conclusion would also be consistent with basic expectation given the consistency of the isomer toxicity differences throughout the range of AChE-inhibiting compounds shown in Table 1.

Stereoc hemical variations in biologically active molecules often play a major role in the effectiveness of drugs or the specificity of pesticides. Toxin molecules such as Botulinum toxin and other toxins fused to antibodies are used as therapeutics. One means of the preparation of pure stereoisomers is through the preferential enzymatic degradation of one stereoisomer, leaving most of the other isomer intact. In these investigations, the stereospecificity of the wild-type enzymes permitted the preparation of a pure sample of (−)GF by preferentially degrading the (+)GF stereoisomer and extracting the resulting product. Polarimetry established the identity of the remaining isomer, which then allowed the assignment of peaks in the isothermal GC method that was developed to resolve the two GF isomers and permit their chromatographic identification either alone or together. A chromatographically pure preparation of (+)GF was made using the GWT mutant of PTE to preferentially degrade the (−) stereoisomer. Polarimetry analysis confirmed the chiral identity of the extracted and concentrated product. Thus, preparations of either GF isomer could be made from the racemic mixture by using the enzyme with the corresponding stereospecificity.

The enzyme-catalyzed hydrolysis of G-type chemical nerve agents has been investigated for several different potential applications, including surface or personnel decontamination and the use of encapsulated enzymes as *in vivo* scavengers or catalytic treatments for nerve agent poisoning. For instance, the protection of mice from organophosphate intoxication was strikingly enhanced when *in vivo* circulating encapsulated enzymes were used in conjunction with 2-pyridinealdoxime methyl chloride (2-PAM) and/or atropine [32]. For this application, it is important to consider that toxic concentrations of agent in the blood would be orders of magnitude below the highest concentrations at which stereospecificity is observed. Therefore, it would be critical to use enzymes that are active on the toxic isomers at these low concentrations.

When used on higher concentrations of agent such as could be encountered in surface decontamination applications, the fluoride product, which was shown here to be a necessary and sufficient condition for GF racemization, might catalyze racemization of the substrate and thereby facilitate complete degradation of the contaminant. Alternatively, an aqueous decontamination solution could be augmented with millimolar concentrations of NaF to ensure the racemization reaction occurs.
There are also some highly toxic agents such as GV (2-dimethylaminoethyl N,N-dimethylphosphonamidofluoridate) that are largely refractory to traditional atropine/reactivator treatments [33]. In these cases, enzymes offer a potential alternative treatment regime. In the cases where these agents have chiral centers, it will be important to evaluate the enzyme activity on all isomers, particularly those with the greater toxicity.

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